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INTERACTION OF MALAYSIAN SERA WITH *PLASMODIUM VIVAX* SPOROZOITE ANTIGEN

MELIN DA LEE, DON R. DAVIS, W. RIPLEY BALLOU,* GAIL FOLENA-WASSERMAN† AND GEORGE E. LEWIS

*Malaria Research Group, United States Army Medical Research Unit, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia, *Department of Immunology, Walter Reed Army Institute of Research, Washington, DC 20307, and †Department of Protein Biochemistry, SK&F Laboratories, Swedeland, Pennsylvania*

Abstract. A seroepidemiologic survey of *Plasmodium vivax* and *Plasmodium falciparum* transmission was conducted in 94 Orang Asli children and adults. The prevalence of malaria was 46% in this population, and infections due to *P. vivax* and *P. falciparum* occurred with equal frequency. Multi-species infection was common, particularly in children <10 years of age. Circumsporozoite (CS) antibodies to *P. vivax* were detected by ELISA, using the recombinant protein $NS1_{81}V20$, in sera from 53–95% of all subjects in this study. The specificity of reactivity to $NS1_{81}V20$ was confirmed by immunofluorescence using air-dried sporozoites. CS antibodies to *P. falciparum* were present in <50% of the population <30 years of age. These data support further testing of this protein as a candidate vivax vaccine.

Plasmodium vivax is the major cause of relapsing malaria in most malarious regions except sub-Saharan Africa. Immunization with irradiated *P. vivax* sporozoites has been shown to induce protection against experimental sporozoite challenge and to elicit antibodies directed against immunodominant epitopes of the circumsporozoite (CS) protein that mediates in vitro reactions thought to correlate with protective immunity.[1] Cloning and sequencing of the gene encoding the CS protein of *P. vivax* has led to the development of subunit vaccines.[2–4] As in *P. falciparum*, the CS protein of *P. vivax* consists of immunodominant epitopes repeated in tandem flanked by nonrepeating sequences, some of which are conserved among malaria species. We demonstrate that $NS1_{81}V20$, a recombinant fusion protein expressed in *E. coli* and containing the entire central repeat portion of the vivax CS protein, is specifically recognized by sera obtained from persons with lifetime exposure to *P. vivax* sporozoites. Our data indicate that the immunodominant CS epitopes of *P. vivax* sporozoites are highly immunogenic for humans and suggest that $NS1_{81}V20$ is a suitable candidate for further clinical trials.

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MATERIALS AND METHODS

Human sera

Monthly malariametric surveys were conducted in the Pos Legap area of Perak State, Malaysia, August–November 1986. The aboriginal population living in this region employs little, if any, routine malaria prophylaxis or effective malaria control measures. Consistent population point prevalences of 38–40% for infection with *Plasmodia* of one or more species were found. Subsequent surveys substantiated year-round transmission of *P. falciparum*, *P. vivax*, and *P. malariae*. To test the hypothesis that prolonged natural exposure to *P. vivax* sporozoites induces antibodies that react with $NS1_{81}V20$, we randomly selected 94 serum samples from among 595 subjects aged 6 months–53 years. Nonimmune sera obtained from 30 healthy U.S. servicemen with no known exposure to malaria served as normal controls.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISAs) were performed as described[5] except that plates were coated with $NS1_{81}V20$ at a concentration of 0.1 μg/well for the detection of anti-

TABLE 1

Distribution and prevalence of malaria by species among 94 Orang Asli as determined by microscopic evaluation of thick smears of peripheral blood

Age (years)	n	Pf	Pv	Pm	Pf/Pv	Pv/Pm	Pf/Pm	Pv/Pf/Pm	Prevalence
≤4	17	2	0	0	3	3	3	2	76%
5–9	19	3	2	0	3	0	0	2	53%
10–19	15	0	4	0	2	0	0	0	40%
20–29	15	1	3	0	2	0	0	0	40%
30–39	16	3	1	1	0	1	0	0	38%
>40	12	2	1	1	1	0	0	0	41%
	94	11	10	2	11	4	3	4	46%

n = Number tested in each age group.
Pf = *P. falciparum*, Pv = *P. vivax*, Pm = *P. malariae*.

bodies to the *P. vivax* CS protein, and with $R32tet_{32}$ for the detection of *P. falciparum* CS antibodies. Sera were diluted 1:100, and the absorbance of sera in wells without antigen was subtracted from the absorbance of sera in wells with antigen to control for background reactivity. Positive reactions for both vivax and falciparum assays were defined as an optical density (OD) exceeding the mean plus 3 standard deviations (SD) of the 30 normal control sera (0.115, $NS1_{81}V20$; 0.101, $R32tet_{32}$).

Immunofluorescent assays

We selected 30 immune sera for characterization by immunofluorescent assays (IFAs). Sera diluted 1:40 were assayed using air-dried salivary gland sporozoites from the Chesson strain of *P. vivax* and the NF54 strain of *P. falciparum* as previously described.[6] Results were graded 0–4+, with 0 indicating no fluorescence and 4+ indicating intense fluorescence along the entire sporozoite. Antibodies against malaria blood stage antigens were assayed using *P. cynomolgi* parasitized monkey erythrocytes (to detect antibodies against *P. vivax*) or *P. falciparum* infected human erythrocytes.[7]

Peripheral blood smears

Peripheral blood smears were obtained and prepared from each of the 94 individuals in the study at the same time that sera were drawn. For each specimen, 200 oil immersion fields (1,000×) of a Giemsa stained thick smear were examined by an experienced microscopist.

RESULTS

Malaria was hyper- to holoendemic in the study population (Table 1). Similar rates were found for *P. vivax* (30/46, 65%) and *P. falciparum* (29/46, 63%). Multi-species infections were more common in children <10 than in older subjects (16/22 vs. 6/22, $\chi^2 = 8.712$, $P < 0.01$), as were infections due to *P. malariae* (10/13 vs. 3/13, $\chi^2 = 5.254$., $P = 0.02$).

The proportion of subjects having CS antibodies and the mean OD by ELISA of those sera to both *P. vivax* and *P. falciparum* CS proteins increased with age (Figs. 1, 2). The mean OD of *P. vivax* CS antibodies was highest in subjects ≥30 years of age. These individuals also had a lower incidence of *P. vivax* infections compared to subjects <30 years of age (4/28 vs. 26/66, $\chi^2 = 6.52$, $P = 0.01$). Despite the fact that equal numbers of individuals in the total population were infected with *P. falciparum* and *P. vivax*, more than 50% of the subjects in each age group had antibodies to *P. vivax* CS protein, whereas seroconversion of ≥50% of the subjects to *P. falciparum* CS protein occurred only after age 30. As shown in Figure 3, the results of ELISA using the recombinant antigens correlated well with IFA using air-dried sporozoites ($r = 0.743$, *P. vivax*; $r = 0.621$, *P. falciparum*). Blood stage antibodies to both *P. falciparum* and *P. vivax* were present in all individuals, and titers increased with age (Fig. 4).

DISCUSSION

This seroepidemiologic study identified an aboriginal Malaysian population with a high prevalence of *P. vivax* and *P. falciparum* infections.

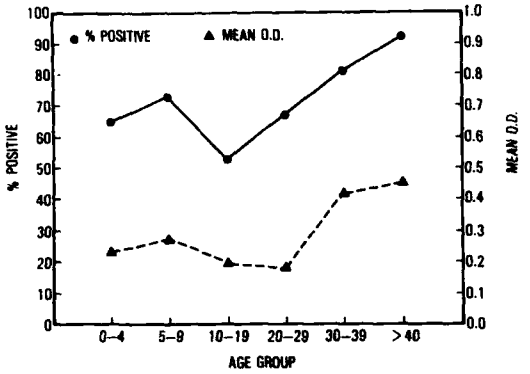

FIGURE 1. Antibody responses to *P. vivax* CS antigen as determined by ELISA using $NS1_{81}V20$ as capture antigen. The percent of positive sera (●) and the mean OD (▲) are shown for each age group.

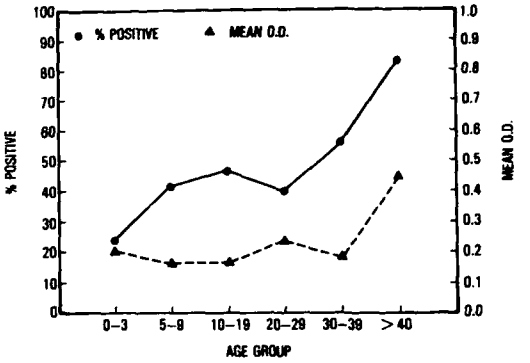

FIGURE 2. Antibody responses to *P. falciparum* CS antigen as determined by ELISA using $R32tet_{32}$ as capture antigen. The percent of positive sera (●) and the mean OD (▲) are shown for each age group.

Although there are numerous reports describing CS antibody responses to the immunodominant repeat epitopes of *P. falciparum*, similar data for *P. vivax* responses are limited. A better understanding of the human immune response to this CS protein will be important to sporozoite vaccine development for *P. vivax*. Sera from a majority of these subjects reacted specifically by ELISA with the recombinantly produced *P. vivax* CS protein $NS1_{81}V20$. This antigen, produced in *E. coli*, is a highly purified protein consisting of 81 amino acids from the nonstructural protein 1 of influenza A fused N-terminal to the entire immunodominant central repeat portion (180 amino acids) of the *P. vivax* CS Protein (G. F. Wasserman, personal communication). Preclinical studies with the vaccine demonstrated that immune sera from mice, rabbits, and non-human primates react with intact *P. vivax* sporozoites by IFA (W. R. Ballou, personal communication). These data, together with the high proportion of subjects in this human population having antibodies that react with $NS1_{81}V20$, indicate that important epitopes on the molecule have not been adversely affected either by expression in a prokaryotic system (*E. coli*) or by the presence of the NS1 portion of the fusion protein. Furthermore, the absence of nonspecific reactivity to $NS1_{81}V20$ in 30 normal control sera indicates that antibodies to NS1 are not common and suggests that the possibility of carrier mediated suppression of antibody responses to the CS repeats will be unlikely. These results are consistent with data indicating that convalescent sera from influenza A patients fail to react with $NS1_{81}V20$ by ELISA (D. G. Gordon, Department of Immunology, Walter Reed Army Institute of Research, Washington, DC, personal communication). Antibody responses to the *P. falciparum* CS antigen are consistent with those of similar studies conducted elsewhere demon-

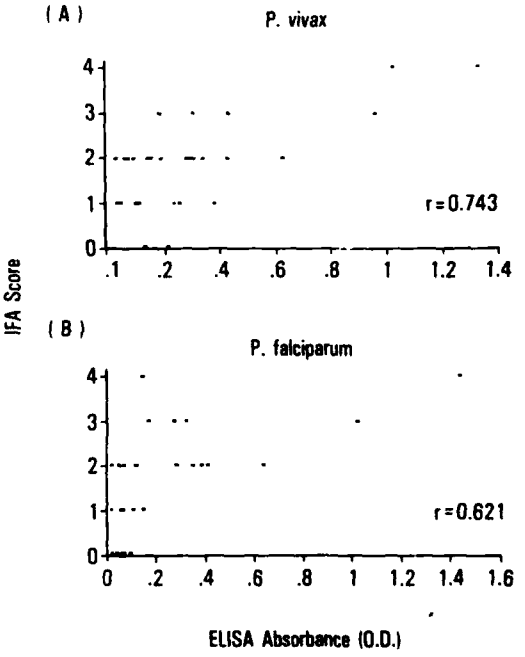

FIGURE 3. Scattergram showing the relationship between ELISA OD and IFA using air-dried sporozoites of *P. vivax* (**A**) and *P. falciparum* (**B**). The correlation coefficients ELISA activity and IFA score are shown.

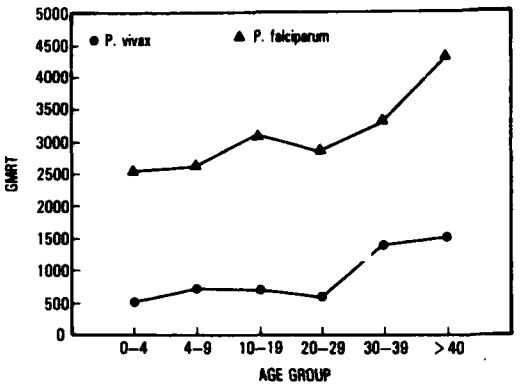

FIGURE 4. Reciprocal geometric mean titers (GMRT) of blood stage antibodies to *P. falciparum* (●) and *P. vivax/P. cynomolgi* (▲) antigens as determined by IFA. Positive responses were defined as those with RGMT ≥ 1:40.

strating an age-dependent acquisition of CS antibodies in a majority of exposed individuals.[8–10]

Despite nearly equal prevalence rates for *P. falciparum* and *P. vivax* malaria, a higher proportion of the study subjects had antibodies to *P. vivax* CS protein than had *P. falciparum* CS antibodies. This was true for all age groups. One possible explanation is that *P. vivax* sporozoites may be inherently more immunogenic for this population than are *P. falciparum* sporozoites. While we cannot exclude differences in antigenic exposure (i.e., greater numbers of sporozoites injected or prolonged exposure to CS proteins during the exoerythrocytic stages of *P. vivax*) higher seroconversion rates to *P. vivax* CS repeat epitopes may reflect less genetic restriction of immune response to this protein as compared to the CS repeats of *P. falciparum*.[10] This may be due to the larger number of amino acids per repeat (9 vs. 4) or the presence of sequence heterogeneity [GlnProAlaGlyAspArgAla (Ala/Asp) Gly] in 50% of the *P. vivax* repeats.[2] Alternatively, the *P. vivax* CS protein may contain more widely recognized T cell epitopes in the nonrepeat flanking regions than those that have been defined for *P. falciparum*.[11]

Although the mean OD of *P. vivax* CS antibodies increased with age, there was a lower prevalence of *P. vivax* infections after age 30, perhaps due to low parasite densities not detected by peripheral blood smears. This study does not suggest that CS antibodies are protective against natural transmission of *P. vivax* sporozoites. This study has, however, identified a population at high risk for *P. vivax* malaria that may be suitable for further analysis of protective immune responses.

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